MLT - Practical (old) Rec:-

Objective: To observe the transmittance and absorbance of the complementary color light by the substances and effects of concentration on these two phenomenon.

Theory

Spectrophotometry is a procedure that is frequently utilized in biological laboratories. Probably the most common application in biology of this technique is in the measurement of the concentration of a compound in solution.

Many kinds of biological substances absorb visible light (400 to 700 nm) selectively and, as a result, appear colored. Substances that do absorb visible light are called pigments. Other substances do not absorb light of visible wavelengths and appear colorless. We can study colorless compounds in two different ways.

- One is that they can often react with other substances to form colored derivatives that we can see and measure.
- Another is that colorless compounds usually absorb light in the region of the spectrum that is not visible to the naked eye. Absorption of this light can be measured, even if we cannot observe it unaided.

Using a spectrophotometer, which measures the absorption by a solution of light of specific wavelengths (visible or not), allows us to determine concentration.

A second application of spectrophotometry is the determination of the absorption spectrum of a compound.

Both of these can be applied to colorless as well as colored solutions since a spectrophotometer can measure absorbance of light that we cannot see.

Absorption spectra and color

Regardless of whether a solution is colorless or colored, the wavelength(s) absorbed are unique. A solution of a particular compound, such as hemoglobin, always absorbs light of specific wavelengths and reflects light of other wavelengths. For every compound, if you measure the proportion of light absorbed, for any wavelength, you will always get the same answer. This is the basic idea of the absorption spectrum of a specific compound, which is the proportion of light absorbed for each wavelength of the spectrum.

The wavelength(s) absorbed by a substance in the visible part of the spectrum is complementary to the color that we perceive. The "color" is a function of human perception, but absorption of specific wavelengths of light is a function of molecular interaction with light.

If a substance absorbs blue and red light, but not green light, we will see it as green since that is the only light that reaches us from that substance. Therefore, when you look at the absorption spectrum of a green solution, it should show low absorbance of wavelengths in the green part of the spectrum but high absorption of light in the red and blue parts of the visible spectrum.

While the "color" of a substance depends on the person observing it, absorption of specific wavelengths depends on the molecular structure of the substance. This allows qualitative analysis (i.e., identification) of some substances by determination of the absorption spectrum.

The wavelength of light absorbed the most is known as (λ_{max}).

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Procedure:

1. Prepare 1mg/ml solution of the given color pigment.
2. Make dilution as given below in 5ml solution.
3.

Dilution#	Blank	Dil-1	Dil-2	Dil-3	Dil-4
Solution (ml)	0	5	4	3	1
DH_2O (ml)	5	0	1	2	4
%T					
Absorbance					

4. Select the wavelength complementary to the color of solution.
5. Prepare the spectrophotometer by setting Absorbance to zero.
6. Record the transmittance and absorbance of different dilution.
7. Draw a line graph with Dilution at X-axis and %T at Y-axis.

Infer the effects of dilution on transmittance and absorbance of selected wavelength:

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Objective: To determine the absorption maxima (λmax) by performing the wavelength scanning of different solution.

Theory

Lambda max (λmax) refers to the wavelength along the absorption spectrum where a substance has its strongest photon absorption. This absorption maxima is characteristic of each component depends on its chemical characteristics.

A scanning spectrophotometer operates on the same principles as a Spectronic 20™. They both can be used for qualitative and quantitative analysis.

A scanning spectrophotometer's main advantage over the Spectronic 20™ is that the scanning spectrophotometer can rapidly scan a range of wavelengths and record absorbance at each wavelength.

Procedure:

1. Prepare 10ml solution of each given color pigment at concentration of 1mg/ml.
2. Mix the pigment to dissolve as much as possible.
3. Filter the solution through Whatman No.1 filter paper.
4. Obtain clean plate reader wells.
5. Place water in first well, this will serve as our blank.
6. Place each filtered solution in triplicate in wells.
7. Make your plate scanner ready for the absorption; place the plate holding the well in the reader.
8. Scan the plate at 350nm wavelength.
9. Note the absorption in the blank and solution containing wells in the chart given below.
10. Repeat steps 8 and 9 with each given wavelength.
11. Subtract the reading of blank well from the solution containing wells in each corresponding light wavelength.
12. Average the absorption reading for each solution.
13. Draw a line graph for each solution to find at wavelength the absorption was highest (λmax).

Samp#	W1							

Results:

	λ_{max}
Solution 1	
Solution 2	
Solution 3	

Note: note the wavelength of light at which the solution shown the lowest absorption and how it corresponding to the color of solution.

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Objective. Produce standard curve for the red pigment solution and utilize it to measure the solution with unknown concentration of same pigment.

Theory:

Measuring concentration

The basis of this application of spectrophotometry is that the proportion of light that is absorbed by a solution of a particular compound is a function of the concentration of that compound. This allows for a quantitative analysis of concentration of a substance from the Beer-Lambert relationship.

$A = \varepsilon l c$ OR $c_{molar} = A / \varepsilon l$

where A is absorption, ε is molar absorptivity, l is the path length (which is constant 1cm so can be omitted in the formula in most cases), c stand for concentration.

A spectrophotometer will direct light of a specific wavelength on your solution. This light is the incident light (I_0). The light that passes through the solution is the transmitted light (I).

$T = I / I_0$ OR $T\% = (I / I_0) \times 100$

The absorbance (A) of the solution is the log of the ratio of these two measures.

$A = \log_{10} I_0 / I$ OR $A = -\log_{10} T$

In order to apply the Beer-Lambert equation you must know the Molar Absorption Value (E) for the substance (compound) and wavelength you are using. This is defined as the Absorbance of a 1 M solution so it can be measured easily by the obvious - reading Absorbance of a 1 M solution.

Standard Curve:

The extinction coefficient (e) is characteristic of a given substance under a precisely defined set of conditions, such as wavelength, solvent, and temperature. This can be used to measure the concentration of unknown solution using the Beer-Lambert relationship. However, for substances with strong absorbance, the Absorbance of a l M solution is too great to read in an analytical instrument. The measured extinction coefficient also depends partially on the characteristics of the instrument used. For these reasons, predetermined values for the extinction coefficient usually are not used for quantitative analysis. In this case a standard curve is produced by measuring Absorbance of a number of dilute solutions, each of known concentration. *When Absorbance is plotted against concentration the slope of the line is the relationship between concentration and Absorbance*. This is the E value (E=A/C, if L=1) so the slope of the standard curve gives you E. Once you have measured E, you can find the concentration of any solution (of that compound) by measuring Absorbance at the *same* wavelength used to calculate E.

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Procedure

1. Prepare 0.2mg/ml solution of given red pigment (RP)
2. Prepare 6 tubes according to the chart given below to prepare the solution of given concentrations.
3. Prepare the Spectronic 20 for the reading by zeroing the absorption using the blank tube.
4. Record the Absorption for the different concentration of methylene blue.
5.

Std#	Conc.	RP (0.2mg/ml) ml	D.H_2O ml	A_{590}
1	0.2			
2	0.16			
3	0.12			
4	0.8			
5	0.4			
6	0 0			

6. Plot the absorbance at Y axis and concentration of standard at X axis.
7. Calculate the slope and intercept using the scatter plot graph equation.
8. Record the Absorption for the methylene blue solution of unknown concentration.
9. Calculate the concentration of unknown samples using the slope equation.

Results:

Sample#	A_{590}	Conc.
1		
2		
3		

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Objective: Produce standard curve for the methylene blue solution and utilize it to measure the methylene blue solution with unknown concentration.

Theory.

Measuring concentration

The basis of this application of spectrophotometry is that the proportion of light that is absorbed by a solution of a particular compound is a function of the concentration of that compound. This allows for a quantitative analysis of concentration of a substance from the Beer-Lambert relationship.

$A = \varepsilon lc$ OR $c_{molar} = A / \varepsilon l$

where A is absorption, ε is molar absorptivity, *l* is the path length (which is constant 1cm so can be omitted in the formula in most cases), *c* stand for concentration.

A spectrophotometer will direct light of a specific wavelength on your solution. This light is the incident light (I_O). The light that passes through the solution is the transmitted light (I).

$T = I / I_O$ OR $T\% = (I / I_O) \times 100$

The absorbance (A) of the solution is the log of the ratio of these two measures.

$A = \log_{10} I_O / I$ OR $A = -\log_{10} T$

In order to apply the Beer-Lambert equation you must know the Molar Absorption Value (E) for the substance (compound) and wavelength you are using. This is defined as the Absorbance of a 1 M solution so it can be measured easily by the obvious - reading Absorbance of a 1 M solution.

Standard Curve:

The extinction coefficient (e) is characteristic of a given substance under a precisely defined set of conditions, such as wavelength, solvent, and temperature. This can be used to measure the concentration of unknown solution using the Beer-Lambert relationship. However, for substances with strong absorbance, the Absorbance of a 1 M solution is too great to read in an analytical instrument. The measured extinction coefficient also depends partially on the characteristics of the instrument used. For these reasons, predetermined values for the extinction coefficient usually are not used for quantitative analysis. In this case a standard curve is produced by measuring Absorbance of a number of dilute solutions, each of known concentration. *When Absorbance is plotted against concentration the slope of the line is the relationship between concentration and Absorbance*. This is the E value (E=A/C, if L=1) so the slope of the standard curve gives you E. Once you have measured E, you can find the concentration of any solution (of that compound) by measuring Absorbance at the *same* wavelength used to calculate E.

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Procedure

10 Prepare 6 tubes according to the chart given below to prepare the solution of given concentrations
11 Prepare the Spectronic 20 for the reading by zeroing the absorption using the blank tube
12 Record the Absorption for the different concentration of methylene blue.
13

Std#	Conc.	MB (5mg/ml) ml	D H_2O ml	A_{664}
1	0.5	0.3	2.7	
2	1.0	0.6	2.4	
3	1.5	0.9	2.1	
4	2.0	1.2	1.8	
5	2.5	1.5	1.5	
6	3.0	1.8	1.2	

14. Plot the absorbance at Y axis and concentration of standard at X axis.
15. Calculate the slope and intercept using the scatter plot graph equation.
16. Record the Absorption for the methylene blue solution of unknown concentration.
17. Calculate the concentration of unknown samples using the slope equation.

Results:

Sample#	A_{664}	Conc.
1		
2		
3		

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